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Metabolic Effects of Epinephrine in the Isolated, Perfused Rat Heart

I. DISSOCIATION OF THE GLYCOGENOLYTIC FROM THE METABOLIC STIMULATORY EFFECT*

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The metabolic effects of epinephrine on the carbohydrate metabolism of muscle tissues have been extensively studied both *in vivo* and *in vitro*, but conflicting evidence makes it uncertain whether decreased glucose utilization by muscle contributes significantly to the hyperglycemia produced by this hormone (1–4). An increased conversion of phosphorylase *b* to *a* and increased glycogenolysis are widespread effects of epinephrine, which have been observed in many tissues (1, 5–7). In liver, glycogen breakdown leads to an increased glucose output, while in skeletal muscle, lactate is the main product, and may subsequently be used as a metabolic fuel (8). A net decrease of glycogen has not always been observed with heart muscle *in vivo*, however, the skeletal muscle appears to be more sensitive than cardiac muscle to the glycogenolytic effect of epinephrine (1, 9–11).

It is well established that the addition of epinephrine to rat diaphragm preparations *in vitro* results in a decreased glucose uptake (1, 5, 12), and administration of epinephrine to anesthetized animals *in vivo* has been shown to cause an intracellular accumulation of glucose and an increase of glucose 6-phosphate levels in skeletal and cardiac muscle (10, 13, 14). Since glucose 6-phosphate is a well known inhibitor of hexokinase, these results suggest that glucose phosphorylation may be decreased as a secondary effect of glycogenolysis. This conclusion is not in agreement with earlier work, which showed that epinephrine increased the oxygen consumption, and the glucose and lactate utilization, of the dog heart-lung preparation (15–17). It has also been shown that epinephrine increases the carbohydrate utilization of exercising human subjects (18). Unlike the glycogenolytic action, the inotropic action of epinephrine on skeletal muscle *in vitro* is manifested only during electrical stimulation (19), and resting cardiac or skeletal muscle fails to respond to epinephrine with an increased oxygen consumption (20–22). The spontaneously contracting perfused rat heart and the isolated cat papillary muscle, however, exhibit an increased oxygen consumption with epinephrine (23, 24), which in the latter preparation has been shown to parallel the increase of contractile force (24).

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These observations suggest that the effect of epinephrine on glucose uptake in muscle may be determined by the ability of the tissue to respond to the inotropic stimulus, and prompted a study of the metabolic effects of epinephrine on the isolated, perfused rat heart. The data presented in this paper describe the effects of epinephrine and other catecholamines on the uptake and metabolism of glucose and acetate, and the temporal and dose-response relationships between the glycogenolytic and metabolic effects of epinephrine. The effects of epinephrine on intracellular levels of glucose, hexose phosphates, and adenine nucleotides have been reported briefly (25) and will be described in detail in a subsequent paper.

EXPERIMENTAL PROCEDURE

Perfusion Techniques—Male rats of the Sprague-Dawley strain weighing between 200 and 280 g were used. All animals had unrestricted access to food and water. The rats were anesthetized with a 50% O_2-50% CO_2 gas mixture, and the heart was removed and mounted on a glass cannula as described previously (26). In the experiments in which the heart was frozen after perfusion, it was mounted on a 18-gauge stainless steel needle. The perfusion apparatus was based on the design of Bleehen and Fisher (27) with the exception that a water-jacketed, coarse, 20-mm fritted glass filter was used as a combined filter and upper reservoir. With this type of filtration, flow rates of 5 to 8 ml per minute were maintained for a 60-minute perfusion period. The basic perfusion medium was Krebs-Ringer-bicarbonate buffer (28) containing 1.27 mM calcium, and the perfusion temperature was 37°. The hearts were preperfused for 15 minutes with medium containing 5 mM glucose prior to transfer to the recirculation apparatus. A single large water-jacketed reservoir (500 ml) served four hearts during preperfusion, and the fluid was oxygenated with 95% O_2-5% CO_2 through a fritted glass gas dispersion tube. This period of preperfusion was important in order to obtain uniform metabolic results during the experimental perfusion, and it ensured the washing out of epinephrine released during preparation of the heart (29, 30).

The recirculation apparatus contained 10 to 20 ml of fluid, and samples were removed at intervals, as indicated in the figures and tables. When the perfusate contained ^{14}C-glucose or ^{14}C-acetate, the $^{14}CO_2$ produced over successive 15-minute intervals was trapped by passing the effluent gas mixture from the heart perfusion apparatus through sodium hydroxide. Counts present as ^{14}C-bicarbonate were determined by volatilizing the CO_2 in an

aliquot of perfusate sample (made alkaline by the addition of 2 volumes of 0.1 N NaOH) into sodium hydroxide with acid. The total $^{14}CO_2$ production was obtained from the sum of the counts appearing directly as CO_2 in the gas phase, and those appearing as bicarbonate in the medium. A second aliquot of the alkaline perfusate sample was counted to obtain the total counts in the medium. Radioactive solutions were counted at 4° in a Nuclear-Chicago liquid scintillation spectrometer by adding a 0.5-ml sample to 15 ml of a dioxane-naphthalene solvent made up as follows: 100 g of naphthalene, 40 g of powdered silicic acid (Cab-O-Sil, obtained from Raw Materials Company, Boston), 7 g of 2,5-diphenyloxazole, and 0.3 g of *p*-bis-2′-(5′-phenyloxazolyl)benzene dissolved in 1 liter of dioxane. The counting efficiency was obtained by adding ^{14}C-toluene as an internal standard and was 56 to 60%. Counts were converted to micromoles of glucose (or acetate) equivalents by dividing by the specific activity of glucose (or acetate). This method of perfusion offers advantages over the closed perfusion system described by Opie, Shipp, Evans, and Leboeuf (31) in that $^{14}CO_2$ production may be monitored over short intervals of time; no assumptions are made about the distribution of CO_2 in the gas and liquid phases, and the pH is maintained constant by continuous equilibration of the perfusate with the oxygenating gas mixture of 95% O_2-5% CO_2.

At the end of perfusion, the hearts were either frozen by clamping between two blocks of aluminum cooled in liquid nitrogen (32), or flushed through with 4 ml of cold 0.9% NaCl at 0°, blotted, and divided into two parts for glycogen and dry weight determinations. Frozen hearts were powdered in a percussion mortar cooled in solid carbon dioxide, and weighed samples of the powder were taken for glycogen, dry weight, and phosphorylase determinations.

Materials—Epinephrine chloride, norepinephrine chloride, and isoproterenol bitartrate (Parke, Davis and Company) were suitably diluted with perfusate, and 0.3 ml of the fresh stock solution was added to the circulating medium 2 minutes before transfer of the heart, or after 30 minutes of perfusion. In most experiments the final concentration of epinephrine was 0.2 μg per ml. Insulin (Lot 466368, glucagon-free) and dichloroisoproterenol were a gift from Eli Lilly and Company. AMP, NADP, NADH, glucose-1-P, glycogen, and glycerol kinase were obtained from Sigma Chemical Company. Phosphoenolpyruvate (tricyclohexylammonium salt), pyruvate kinase, glucose-6-P dehydrogenase, and lactic dehydrogenase were obtained from C. F. Boehringer and Soehne, GmbH. Glucose-1-P and glycogen were purified as described by Sutherland and Wosilait (33).

Analytical Methods—Glucose was determined by the method of Huggett and Nixon (34), and lactate by the method of Horn and Bruns (35). Weighed samples of powdered or chilled heart were added to 5 volumes of 30% KOH (w/v) per g, wet weight, for polysaccharide separation (36). The precipitated polysaccharide was washed three times with 0.5 ml of 95% ethanol, hydrolyzed with 2 ml of 2 N HCl, and assayed for glucose as above. The rapid freezing technique gave values for heart glycogen about 20% higher than those obtained with chilled hearts. In some experiments 2 ml of medium at the end of perfusion were added to 0.5 ml of 0.6 N $HClO_4$, and after neutralization with 2 N KOH, the sample was estimated for glycerol (37).

Assay of Phosphorylase—The method used was based upon that described by Danforth, Helmreich, and Cori (38), with the following modifications. A sample of powdered heart (150 to 200 mg) was weighed in precooled tubes and homogenized at −30°, with the use of a Teflon pestle, with 1 ml of 60% glycerol (w/v) containing 20 mM NaF and 1 mM EDTA (pH 6.1). The tubes were warmed to 0°, 3 ml of buffer (20 mM NaF, 1 mM EDTA, 20 mM sodium glycerophosphate, and 20 mM cysteine, pH 6.1) at 0° were added, and the mixture briefly rehomogenized. After centrifugation, an aliquot of the extract was diluted 10 times more with a similar buffer containing no EDTA. It was necessary to omit EDTA from the dilution fluid at this stage since it interfered with the color development in the phosphate determination at concentrations greater than 1×10^{-5} M.

For measurement of the total phosphorylase activity, 0.5 ml of the diluted extract was added to 0.5 ml of an assay mixture containing 1% glycogen, 66 mM glucose-1-P, and 2 mM AMP. After 5, 15, and 30 minutes of incubation at 30°, 0.2-ml aliquots were removed and added to 3 ml of 0.025 M acetate buffer, pH 4. For estimation of phosphorylase *a* activity, AMP was omitted from the assay mixture. Inorganic phosphate released by phosphorylase action was measured according to the method of Lowry and Lopez (39). The reaction was linear over the 30-minute incubation period, and incubations in the absence of glucose-1-P or glycogen indicated the absence of phosphatase activity. The reaction mixture, after protein precipitation with 0.6 N $HClO_4$ followed by neutralization, was found to contain no glucose-6-P,[1] which showed the absence of phosphoglucomutase activity. The dilution of the heart extract (approximately 400 ml per g of heart, dry weight) was sufficiently great that the concentration of endogenous AMP in the phosphorylase *a* reaction tubes was well below the K_m for phosphorylase *b* activation (30, 40).

RESULTS

Effects of Epinephrine on Glucose Metabolism—The glucose uptake of control hearts perfused with Krebs' bicarbonate medium containing 5 mM glucose was 51 μmoles per g, dry weight, over a 30-minute perfusion period (Table I). Lactate production accounted for about 12% of the glucose uptake and glycogenolysis was not observed. Epinephrine (0.2 μg per ml) in the absence of EDTA produced a 2-fold increase of glucose uptake, a 5-fold increase of lactate production, and a 25% decrease of heart glycogen. In these experiments, the rate of glucose uptake decreased with time after the first 10 minutes and suggested rapid inactivation of the circulating epinephrine. The oxidation of epinephrine is known to be catalyzed by traces of heavy metal ions, and Table I shows that 5×10^{-5} M EDTA substantially increased the effect of epinephrine on glucose and glycogen metabolism, without affecting the control values. This concentration of EDTA was only sufficient to bind about 2% of the calcium in the modified Krebs-Ringer-bicarbonate buffer, and it was added to the perfusate in all subsequent experiments. Table I also shows that these metabolic effects of epinephrine were antagonized by dichloroisoproterenol. The glycogenolytic effect of 0.2 μg per ml of epinephrine was completely blocked by

[1] Glucose-6-P was assayed by adding a 0.5-ml sample to 2 ml of buffer (40 mM glycylglycine, 12 mM $MgCl_2$, and 0.6 mg per ml of NADP, pH 7.6) and following the change in absorbance at 340 mμ on a Beckman spectrophotometer after addition of glucose-6-P dehydrogenase (5 μg in 0.01 ml).

TABLE I

Potentiation by EDTA and antagonism by dichloroisoproterenol of epinephrine effects on carbohydrate metabolism in perfused rat heart

Hearts were preperfused for 15 minutes with oxygenated medium containing 5 mM glucose, and transferred to recirculation perfusion circuits containing 18 ml of 5 mM glucose solution together with EDTA, dichloroisoproterenol, or epinephrine, as noted below. Samples of perfusate (1 ml) were removed for analysis of glucose and lactate, both initially and after 30 minutes of perfusion. Values shown are means ± standard error of the mean and are expressed as micromoles of glucose equivalents per g of heart, dry weight.

Additions to medium			No. of hearts	Glucose uptake	Lactate formation	Final glycogen content*
EDTA, 5×10^{-5} M	Epinephrine	Dichloroisoproterenol				
	μg/ml			μmoles/g/30 min		
0	0	0	10	51 ± 4	6 ± 3	94 ± 3
0	0.2	0	8	116 ± 8	33 ± 6	70 ± 5
+	0	0	10	50 ± 6	10 ± 3	91 ± 7
+	0.2	0	8	187 ± 10	81 ± 10	59 ± 5
+	0	5	4	58 ± 7	6 ± 3	92 ± 8
+	0.2	0.5	4	127 ± 18	33 ± 12	96 ± 6
+	0.2	5	7	98 ± 7	26 ± 5	97 ± 4

* The mean glycogen content of six hearts at the beginning of perfusion was 98 ± 6 μmoles per g, dry weight.

0.5 μg per ml of dichloroisoproterenol, but the glucose uptake remained significantly elevated above controls ($p < 0.01$) even with 5 μg per ml of dichloroisoproterenol.

The glucose uptake and lactate output of control and epinephrine-treated hearts are shown as a function of time in Fig. 1. Lactate production (expressed as glucose equivalents) was greater than glucose uptake over the first 10 minutes of perfusion with epinephrine, but thereafter the rate of lactate formation decreased sharply, whereas the rate of glucose uptake remained approximately constant.

These results suggested that the lactate appearing in the medium during the early phase of epinephrine action was probably derived from heart glycogen. This was confirmed by measuring the lactate production over successive 30-second intervals after addition of epinephrine to hearts perfused with glucose-free medium. The results of one such experiment are shown in Fig. 2. The rate of lactate formation was very low prior to epinephrine addition at 3 minutes, but it increased 16-fold within 1 minute, and remained elevated for a further minute, before falling rapidly to values 3 to 5 times over the controls. The total lactate production of five hearts perfused under similar glucose-free conditions was 38 ± 3 μmoles per g, dry weight, of glucose equivalents during the 5-minute period following epinephrine addition, compared with a lactate production of 46 ± 3 μmoles per g, dry weight, of glucose equivalents by hearts perfused in the presence of epinephrine and glucose over a similar time interval.

The continuous presence of epinephrine was necessary for the effect on glucose metabolism, as shown by another experiment in which hearts were perfused for 30 minutes in the presence of 0.2 μg per ml of epinephrine and, after a brief washing out period, were transferred to other perfusion circuits containing no epinephrine, and were perfused for a further 30 minutes. The glucose uptake and lactate formation of the hearts during the second ½ hour of perfusion were the same as controls.

Heart Glycogen—The glycogen content of hearts perfused with medium containing 5 mM glucose and 5×10^{-5} M EDTA fell slightly from 119 ± 4 (11 hearts) to 107 ± 6 (6 hearts) μmoles per g, dry weight, during a 30-minute perfusion period (Fig. 3). Epinephrine produced a prompt glycogenolysis, and the glycogen content reached a plateau level of 66 to 69 μmoles per g, dry weight, 3 minutes after the addition of epinephrine. The glycogen disappearance within the first 5 minutes of epinephrine addition (46 to 52 μmoles per g, dry weight) was sufficient to account quantitatively for the lactate production over this time interval.

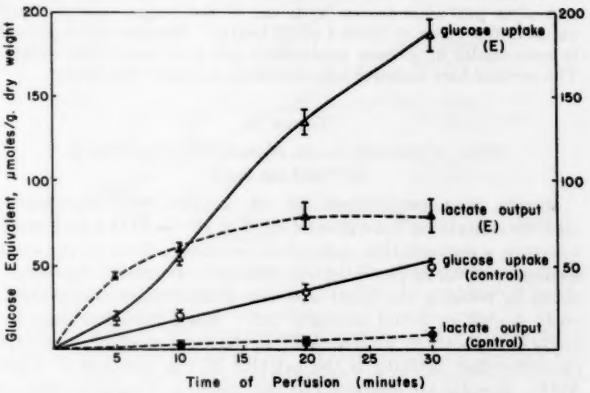

FIG. 1. Rate of glucose uptake and lactate output by control and epinephrine-treated (*E*) perfused rat hearts. The perfusion medium contained 5 mM glucose and 5×10^{-5} M EDTA, and the concentration of epinephrine was 0.2 μg per ml. Values shown are the mean of eight hearts, and are expressed in micromoles of glucose equivalents per g of heart, dry weight. The *vertical bars* indicate two standard errors of the mean.

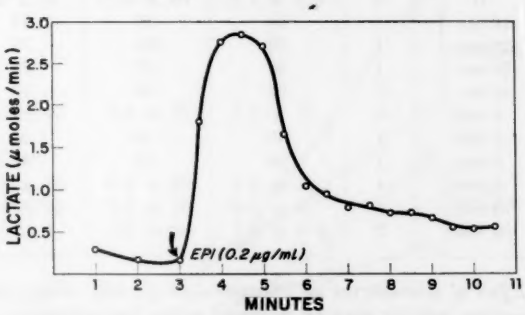

FIG. 2. Effect of epinephrine on the rate of lactate production by rat heart (dry weight, 151 mg) perfused with substrate-free medium. After a 15-minute preperfusion period with medium containing 5 mM glucose, the heart was switched to a substrate-free perfusion medium and the effluent was collected over 1-minute intervals. After 3 minutes, the heart was transferred to another circuit and perfused with substrate-free medium containing 5×10^{-5} M EDTA and 0.2 μg per ml of epinephrine. The effluent was collected over 30-second intervals for a further 7.5 minutes. Lactate output is expressed as micromoles per minute.

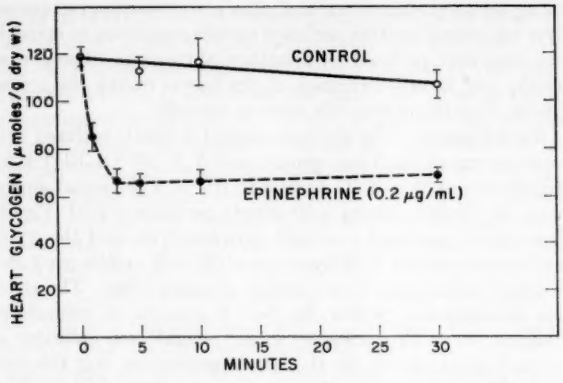

FIG. 3. Effect of epinephrine on glycogen content of the perfused rat heart. Hearts were perfused as described in Table II, and after perfusion frozen with the Wollenberger clamp. Each value is the mean of from 4 to 12 hearts. Results are expressed in micromoles of glucose equivalents per g of heart, dry weight. The *vertical bars* indicate two standard errors of the mean.

TABLE II

Effect of epinephrine on phosphorylase activity of perfused rat heart

Hearts were preperfused for 15 minutes with oxygenated medium containing 5 mM glucose and 5×10^{-5} M EDTA and transferred to a recirculation apparatus containing 15 ml of the same medium and 0.2 μg per ml of epinephrine. Perfusion was terminated by freezing the heart with the Wollenberger clamp previously cooled in liquid nitrogen (32). Basal phosphorylase activity is the activity with no AMP in the assay medium, and total phosphorylase activity is the activity in the presence of 2 mM AMP. Results are expressed as the mean ± standard error of the mean per g of heart, dry weight.

Time of perfusion with epinephrine	No. of hearts	Phosphorylase activity		Phosphorylase *a*
		Basal	Total	
		μmoles P_i/g/min		*% total*
0	6	5.8 ± 1.0	69 ± 5.9	8 ± 0.8
10 sec	1	34	96	35
20 sec	1	82	113	73
30 sec	1	58	87	67
45 sec	1	62	80	78
1 min	5	44 ± 3.7	75 ± 8.6	59 ± 3.2
2 min	1	32	60	53
3 min	1	29	64	45
5 min	4	27 ± 4.6	82 ± 8.0	33 ± 2.7
10 min	4	16 ± 2.0	63 ± 5.7	25 ± 2.0
30 min	3	3.6 ± 0.7	66 ± 12	5 ± 0.7

Effect of Epinephrine on Phosphorylase Activity—Basal phosphorylase activity may be increased either by conversion of *b* to *a*, or by AMP activation of phosphorylase *b*, the K_m of this reaction being 5×10^{-5} M (30). Direct activation of phosphorylase *b* by endogenous AMP was negligible in the present experiments because of the high dilution of the heart extract. Assay of the reaction medium after protein precipitation confirmed that the AMP concentration was less than 10^{-5} M. Hence the ratio of basal phosphorylase activity to total phosphorylase activity probably gives a reasonably accurate measure of the fraction as phosphorylase *a* (30). With control hearts immediately after the 15-minute preperfusion period, phosphorylase was 92% in the *b* form. The phosphorylase *a* activity of control hearts remained low during further perfusion, and after 30 minutes, 5 ± 1.3% (four hearts) of total phosphorylase was in the *a* form. Epinephrine produced a rapid increase of phosphorylase *a*, and a maximum phosphorylase *a* activity of about 70% of total phosphorylase was reached after 20 seconds (Table II). Peak phosphorylase *a* activity was maintained for about 1 minute, and thereafter the percentage of phosphorylase *a* slowly decreased, reaching control levels after 30 minutes of perfusion. The total phosphorylase activity was somewhat variable from heart to heart, but there was no significant difference between groups of hearts after different times of perfusion in the presence or absence of epinephrine.

Effect of Epinephrine Concentration on Glucose Metabolism—As shown in Table III, the lowest concentration of epinephrine which increased glucose uptake above control levels was 0.02 μg per ml. This concentration had no significant effect on lactate production, or heart glycogen content at the end of the 30-minute perfusion period. Glucose uptake followed a linear relation to the logarithm of the epinephrine concentration over the range 0.01 to 0.2 μg per ml, but thereafter showed a tendency to reach a plateau level. Lactate formation was significantly increased above controls with 0.05 μg per ml, but the glycogen content at the end of perfusion was decreased only with 0.2 and 0.5 μg per ml of epinephrine.

Effects of Norepinephrine and Isoproterenol on Glucose Metabolism—Table IV shows that the effect of norepinephrine on the carbohydrate metabolism of the isolated rat heart was the same as that of epinephrine. Isoproterenol was somewhat more potent than epinephrine or norepinephrine, since at concentrations of 0.02 μg per ml, lactate production and glycogenolysis were significantly increased only with isoproterenol. A similar order of potency for the effects of these catecholamines on glycogenolysis and phosphorylase activation has recently been reported with rat hearts *in vivo* (11).

TABLE III

Effect of epinephrine concentration on glucose metabolism of perfused rat heart

Hearts were perfused as described in Table II. Samples of perfusate (1 ml) were removed for analysis of glucose and lactate initially, and after 30 minutes of perfusion. After perfusion, hearts were dropped into ice-cold 0.9% NaCl and a weighed sample of heart was added to 2 ml of 30% KOH (w/v) for glycogen determination (36). Values shown are means ± standard error of the mean and are expressed in micromoles of glucose equivalents per g of heart, dry weight. The mean glycogen content of six hearts at the beginning of perfusion was 98 ± 6 μmoles per g.

Epinephrine	No. of hearts	Glucose uptake	Lactate formation	Final glycogen content
μg/ml		*μmoles/g/30 min*		*μmoles/g*
0	8	50 ± 6	10 ± 3	91 ± 7
0.01	4	48 ± 5	12 ± 2	100 ± 11
0.02	6	86 ± 4*	16 ± 4	90 ± 7
0.05	4	136 ± 8*	48 ± 6*	84 ± 4
0.10	7	155 ± 9*	55 ± 7*	74 ± 8
0.20	7	187 ± 10*	81 ± 10*	59 ± 5*
0.50	6	208 ± 15*	82 ± 10*	60 ± 8*

* $p < 0.01$ *versus* no epinephrine.

TABLE IV

Effect of norepinephrine and isoproterenol on glucose metabolism of perfused rat heart

The experimental conditions were the same as those described in Table III. Values shown are means ± standard error of the mean and are expressed in micromoles of glucose equivalents per g of heart, dry weight.

Drug	Concentration	No. of hearts	Glucose uptake	Lactate formation	Final glycogen content
	μg/ml		μmoles/g/30 min		μmoles/g
Control.....	0	4	35 ± 7	8 ± 3	96 ± 6
Norepinephrine.	0.02	4	80 ± 14*	17 ± 9	83 ± 5
Norepinephrine.	0.20	5	181 ± 10*	75 ± 9*	65 ± 6*
Isoproterenol...	0.01	4	41 ± 8	4 ± 2	87 ± 6
Isoproterenol...	0.02	4	92 ± 16*	39 ± 14*	66 ± 6*
Isoproterenol...	0.10	4	135 ± 16*	75 ± 23*	50 ± 9*

* $p < 0.01$ *versus* control.

Effects of Epinephrine in Presence of Insulin on Glucose Metabolism—Compared with control hearts, insulin produced a 3-fold increase of glucose uptake and a 5-fold increase of lactate production over a 30-minute perfusion period. Epinephrine in the presence of insulin produced a further stimulation of glucose uptake, which was elevated 57% above the insulin controls after 30 minutes (Fig. 4). The rate of glucose uptake was linear in both the presence and absence of epinephrine. Lactate formation increased rapidly over the first 5 minutes of perfusion with the epinephrine-treated hearts, but thereafter little further lactate appeared in the perfusate.

Insulin increased the glycogen content of hearts from 138 ± 4 to 166 ± 6 μmoles per g, dry weight, over the 30-minute perfusion period (eight hearts in each group). Epinephrine in the presence of insulin decreased the glycogen content from 138 ± 4 to 120 ± 6 (eight hearts), 89 ± 12 (four hearts), and 79 ± 4 (four hearts) μmoles per g, dry weight, after 1, 3, and 5 minutes of perfusion, respectively. Insulin thereafter promoted glycogen synthesis, and the mean glycogen content of four hearts after 30 minutes of perfusion in the presence of epinephrine and insulin was 103 ± 5 μmoles per g, dry weight. Lactate formation over the first 5 minutes of perfusion in the presence of epinephrine and insulin was 84 μmoles of glucose equivalents per g, dry weight, compared with a glycogen decrease of 59 μmoles per g, dry weight, over the same time interval. Glycogen was the probable source of most of the lactate in the medium, as in the absence of insulin.

Effects of Insulin and Epinephrine on Metabolism of Uniformly Labeled Glucose-^{14}C—The fact that the increased glucose uptake observed with the perfused rat heart after addition of epinephrine or insulin was associated with an increased glucose oxidation was confirmed by the use of glucose-^{14}C. Insulin alone stimulated $^{14}CO_2$ production 2-fold over a 60-minute perfusion period, and epinephrine (added after 30 minutes) produced a further stimulation of $^{14}CO_2$ production in both the presence and absence of insulin (Fig. 5). There was a lag in the appearance of $^{14}CO_2$ from glucose over the first 15 minutes of perfusion, the values for $^{14}CO_2$ production for the control hearts in the absence of insulin, expressed in glucose equivalents, being 7.7 ± 0.4, 11.1 ± 1.4, 11.5 ± 1.8, and 12.6 ± 1.4 μmoles (six hearts) over consecutive 15-minute intervals. During the first 15 minutes of perfusion with these hearts, 62% of the counts removed from the medium remained in the tissue, while during subsequent intervals, only 20 to 25% of the counts taken up were retained. There was almost no further lag in the $^{14}CO_2$ production when epinephrine was added after 30 minutes of perfusion, showing that the 30- to 60-minute time interval was more suitable than the 0- to 30-minute time interval for a comparison of metabolic rates, since linearity of isotope release had been achieved.

The total metabolic changes of the four groups of hearts shown in Fig. 5 over a 60-minute perfusion period are summarized in Table V, and an approximate evaluation of the major routes of glucose disposal may be made. Apart from oxidation to $^{14}CO_2$, the carbon atoms of glucose may be metabolized to compounds which reappear in the perfusate (*e.g.* lactate and pyruvate) or to

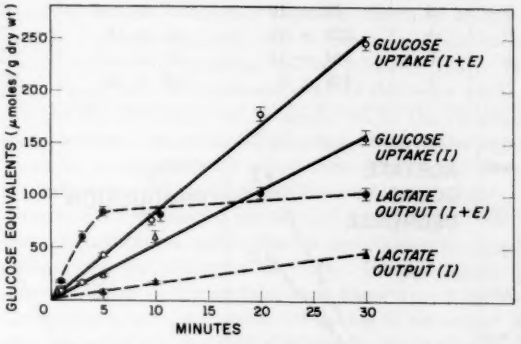

FIG. 4. Effect of epinephrine (*E*) on the rate of glucose uptake and lactate output of rat hearts perfused in the presence of insulin (*I*). The perfusion medium contained 5 mM glucose, 5×10^{-5} M EDTA, and 2×10^{-3} unit per ml of insulin, and the epinephrine concentration was 0.2 μg per ml. Values shown are the means of four or eight hearts, and are expressed in micromoles of glucose equivalents per g of heart, dry weight. The *vertical bars* indicate two standard errors of the mean. △——△, glucose uptake, insulin control; ▲- - -▲, lactate output, insulin control; ○——○, glucose uptake, insulin plus epinephrine; ●- - -●, lactate output, insulin plus epinephrine.

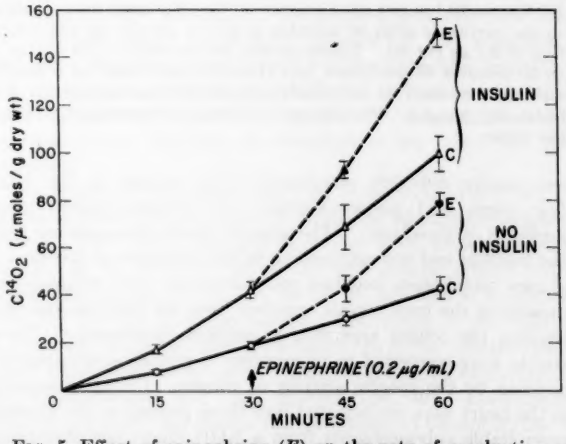

FIG. 5. Effect of epinephrine (*E*) on the rate of production of $^{14}CO_2$ from glucose-^{14}C by rat hearts perfused in the presence and absence of insulin (2×10^{-3} unit per ml). Hearts were perfused as described in Table V. Each value is the mean of 10 hearts up to 30 minutes of perfusion, and thereafter the mean of 5 hearts. Results are expressed as micromoles of glucose equivalents per g of heart, dry weight. The *vertical bars* indicate two standard errors of the mean.

TABLE V

Summary of effects of epinephrine and insulin on metabolism of glucose-^{14}C by perfused rat heart

Hearts were preperfused as described in Table II, and transferred to recirculation apparatuses containing 15 or 20 ml of the same medium and 0.1 μc per ml of glucose-^{14}C. Insulin when added was present at a concentration of 2×10^{-3} unit per ml. Duplicate samples of perfusate (0.5 ml) were taken for analysis and counting initially, and at 15-minute intervals, during the 60-minute perfusion period. Epinephrine was added after 30 minutes from a freshly prepared solution containing 20 μg per ml, made up with perfusate, to give an initial circulating concentration of 0.2 μg per ml. Values shown are means ± standard error of the mean of five hearts, and are expressed as glucose equivalents per g of heart, dry weight.

Insulin	Epinephrine added after 30 min	Glucose uptake	Lactate formation	Medium count decrease	Glucose-^{14}C conversion to			Recovery of isotope
					CO_2	Intermediates	Glycogen	
		μmoles/g/60 min						%
0	0	72 ± 10	5.7 ± 1.4	67 ± 4	43 ± 5	14 ± 2	1.8 ± 0.6	88
0	+	229 ± 10	94 ± 11	150 ± 5	79 ± 5	32 ± 2	9.6 ± 1.1	80
+	0	354 ± 13	78 ± 6	241 ± 14	100 ± 7	54 ± 1	47 ± 4.6	83
+	+	428 ± 6	144 ± 10	261 ± 8	150 ± 6	42 ± 1	26 ± 4.2	84

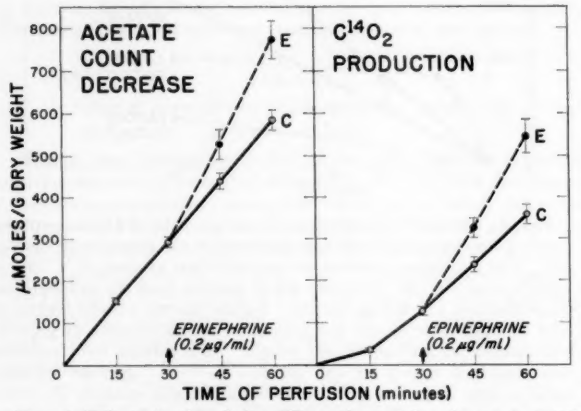

FIG. 6. Effect of epinephrine (*E*) on the uptake of acetate-2-^{14}C and production of $^{14}CO_2$ by the perfused rat heart. Hearts were perfused with medium containing 10 mM acetate, 5×10^{-5} M EDTA, and 0.1 μc per ml of acetate-2-^{14}C. Epinephrine was added to the perfusate after 30 minutes to give a circulating concentration of 0.2 μg per ml. Values shown are the mean of 10 hearts up to 30 minutes of perfusion, and thereafter the mean of 5 hearts, and are expressed as micromoles of acetate equivalents per g of heart, dry weight. *Vertical bars* indicate two standard errors of the mean.

less readily diffusible compounds which remain in the heart (*e.g.* oligo- and polysaccharides, and phosphorylated intermediates of glycolysis). The glucose counts disappearing from the medium and not reappearing in the perfusate as products of glucose metabolism (medium count decrease) were estimated by measuring the total counts removed from the medium and subtracting the counts appearing as medium bicarbonate. These counts were converted to micromoles of glucose equivalents by dividing by the specific activity of glucose. Counts remaining in the heart were fractionated into those present in the alcohol-precipitable polysaccharide from a KOH digest and into those remaining in the alcoholic KOH supernatant. Table V shows that total recovery of the counts disappearing from the medium ranged from 80 to 88%. The difference between the glucose uptake and the decrease of counts in the medium represents the formation of metabolic products (principally lactate) from ^{14}C-glucose which remained in the medium. With glucose alone present in the perfusate, these products accounted for only 7% of the glucose uptake, but they accounted for a much larger proportion of the glucose uptake in the presence of epinephrine or insulin. Table V also shows that epinephrine stimulated a 2-fold incorporation of ^{14}C-glucose into heart intermediates, and a 5- to 6-fold incorporation into glycogen. Insulin increased the incorporation of ^{14}C-glucose into heart intermediates 4-fold, and into glycogen, 26-fold. The addition of epinephrine to hearts perfused in the presence of insulin decreased the incorporation of ^{14}C-glucose into both heart intermediates and glycogen.

Effect of Epinephrine on Acetate Oxidation—The effect of epinephrine on the uptake of acetate-2-^{14}C by the perfused rat heart and its oxidation to $^{14}CO_2$ is shown in Fig. 6. With control hearts, the acetate uptake as estimated by the decrease in counts in the medium was linear over the 60-minute perfusion period. There was a pronounced lag in the appearance of $^{14}CO_2$, which was linear only after the first 30 minutes of perfusion. From 30 to 60 minutes, 78% of the counts disappearing from the medium with control hearts were recovered as $^{14}CO_2$. Epinephrine stimulated acetate uptake and $^{14}CO_2$ production equally, and in the presence of epinephrine, 87% of the counts removed from the medium over the 30- to 60-minute time interval were recovered as $^{14}CO_2$. Only ' μmole per g, dry weight, of acetate-2-^{14}C was incorporated into glycogen, in either the presence or absence of epinephrine, out of a total acetate count decrease of 586 μmoles per g, dry weight, in the absence, and 774 μmoles per g, dry weight, in the presence, of epinephrine. The incorporation of acetate-2-^{14}C into unidentified heart intermediates was large (170 μmoles per g, dry weight) but was not affected by the presence of epinephrine.

Lactate production by the control hearts was very small (5 μmoles per g, dry weight, of glucose equivalents) over the 60-minute perfusion period, and the mean glycogen content at the end of perfusion was 112 ± 6 μmoles per g, dry weight (four hearts). Epinephrine increased the total lactate formation by 62 μmoles per g, dry weight, of glucose equivalents, of which 94% appeared within the first 15 minutes of epinephrine addition. The glycogen content of the epinephrine-treated hearts was decreased to 39 ± 4 μmoles per g, dry weight (four hearts) at the end of the perfusion, indicating that approximately 73 μmoles per g, dry weight, of glycogen disappeared during the 30-minute perfusion period with epinephrine. Thus, in the absence of any

other carbohydrate precursors, lactate appearing in the medium accounted for 85% of the glycogen breakdown under the stimulation of epinephrine.

Effect of Epinephrine on Glycerol Formation—Epinephrine increased glycerol formation by the perfused rat heart. The mean glycerol output of four control hearts perfused for 30 minutes with 5 mM glucose solution was 1.11 ± 0.23 μmoles per g, dry weight. In the presence of epinephrine (0.2 μg per ml), the glycerol production by hearts perfused for 5, 10, and 30 minutes was 1.12 ± 0.10, 2.00 ± 0.18, and 4.84 ± 0.58 μmoles per g, dry weight, respectively (four hearts in each group).

DISCUSSION

Although the positive inotropic action of epinephrine has not been measured in the present investigation, this parameter has been extensively studied in isolated, perfused rat heart (41–43), perfused guinea pig heart (44), isolated cat papillary muscle (24), and the intact dog heart (45, 46), and shown to have a prolonged duration. In the present experiments, heart rates (estimated visually) were elevated above the mean basal rate of 250 beats per minute for the duration of the 30-minute perfusion with epinephrine. Fisher and Williamson[2] (23) observed that the oxygen consumption and flow rate of the perfused rat heart increased immediately after addition of epinephrine and remained elevated for about 30 minutes, and Lee (24) found that the increased force of contraction of cat papillary muscle with epinephrine or norepinephrine was paralleled by an increased rate of oxygen consumption. These and other observations discussed by Moran and Cotten (47) suggest that the augmentation of glucose uptake and oxygen consumption by epinephrine is the result of increased heart work, and may be used as an index of inotropic action. This suggestion is supported by the observation that dichloroisoproterenol, a well known inhibitor of the inotropic action of epinephrine, decreased the glucose uptake of the perfused rat heart in the presence of epinephrine. Relative hypoxia of the tissue, due to an increased oxygen demand uncompensated by an increased oxygen supply, is unlikely to be a factor contributing to the increased glucose uptake with the epinephrine, since direct measurements of the tissue PO_2 indicate adequate oxygenation with both high and low doses.[3]

Previous studies (26, 48) have shown that glucose was poorly utilized by the perfused rat heart, and accounted at most for 24% of the respiratory fuel. The remaining fuel was provided by the oxidation of endogenous noncarbohydrates since the glycogen decrease during perfusion was small. Shipp *et al.* (49) reported that ^{14}C-labeled endogenous lipids were readily oxidized by the perfused rat heart, and a decrease of totally extractable lipid during perfusion with medium containing 5 mM glucose has been observed by Williamson.[4] A similar utilization of endogenous substrates has been observed with incubated rat diaphragm (50). Randle, Garland, Hales, and Newsholme (51) have reported that the perfused rat heart and the isolated rat diaphragm liberate glycerol into the medium, and by analogy with adipose tissue, glycerol output was taken to indicate that endogenous triglyceride had been utilized. The present investigation shows that the increased fuel requirement of the isolated rat heart during epinephrine stimulation was provided partly by a 2- to 3-fold increase in the rate of glucose oxidation, and partly by a 3- to 4-fold increase in the rate of triglyceride utilization, as estimated by glycerol formation. The latter effect may represent a lipolytic action of epinephrine in heart similar to that seen in adipose tissue. The rate of glycerol release as a measure of triglyceride breakdown has to be accepted with caution, however, since it has not been shown that the glycerol was derived from triglyceride rather than from hydrolysis of α-glycerophosphate (52). The possible increased utilization of other endogenous fuels such as amino acids or protein was not investigated, but may prove of interest (1).

With control hearts, acetate was oxidized to $^{14}CO_2$ 3 times faster than glucose per carbon atom, over the 30- to 60-minute perfusion period. Epinephrine increased $^{14}CO_2$ production from acetate-2-^{14}C by 83%. Acetate has been shown to inhibit the utilization of endogenous fuels, and to behave very similarly to free fatty acids as a metabolic fuel for oxidation (53). These results indicate that the increased work of the heart under the stimulation of epinephrine can be supported by the oxidation of a variety of fuels. Oxidation of glucose is of particular interest, and possible mechanisms by which epinephrine may increase glucose utilization will be discussed in a subsequent paper.

The data with ^{14}C-labeled glucose and acetate show that misleading information can be obtained if unsuitable time intervals are chosen for the collection of $^{14}CO_2$. Glucose uptake and decrease of counts in the medium were linear over a 60-minute perfusion period, but there was a marked lag in the rate of $^{14}CO_2$ appearance, which did not approach linearity until after the first 15 minutes of perfusion. Acetate oxidation followed a similar pattern. The total recovery of ^{14}C isotope in these experiments varied from 80 to 92%, but about 30% of the counts removed from the medium were recovered in the nonpolysaccharide fraction of the heart, indicating that intermediates of glycolysis, and possibly other compounds, had to approach a uniform specific activity before $^{14}CO_2$ appearance represented the true rate of substrate oxidation. Over 30 to 60 minutes of perfusion, $^{14}CO_2$ production accounted for 79 and 78%, respectively, of the glucose and acetate uptake of control hearts, which indicated complete oxidation of these substrates. The extra acetate removed from the medium during epinephrine stimulation was completely oxidized. However, when glucose was the exogenous substrate, only part of the extra ^{14}C-glucose removed by the heart appeared as $^{14}CO_2$, and incorporation of counts into intermediates which returned to the perfusate or remained in the heart was also increased.

With control hearts, phosphorylase *a* represented 5 to 8% of the total phosphorylase activity during the 30-minute perfusion period. This value compares favorably with values reported recently in the literature for cardiac and skeletal muscle under control conditions (11, 30, 38, 40–43, 45, 46), and is consistent with the low rate of glycogenolysis. Conversion of phosphorylase *b* to *a* in the preparation of the heart extract for phosphorylase assay was minimized by quick freezing of the heart, extraction at low temperatures, and high dilution (38, 40). Epinephrine produced a rapid increase in the percentage of phosphorylase *a* without affecting total phosphorylase activity. The conversion of phosphorylase *b* to *a* during the first few minutes of epinephrine action was accompanied by a rapid decrease of heart glycogen and appearance of lactate in the perfusate. The glycogen content of hearts after 3 minutes of perfusion in the presence of epinephrine reached a steady state, however, despite elevated levels of phosphorylase *a*. Phosphorylase *a* levels subsequently

[2] R. B. Fisher and J. R. Williamson, unpublished observations.
[3] J. R. Williamson, B. Chance, and D. Jamieson, in preparation.
[4] J. R. Williamson, unpublished experiments.

fell, while fuel utilization remained increased. Studies with ^{14}C-glucose indicated that epinephrine stimulated the incorporation of glucose into glycogen during a 30-minute perfusion period, showing that after the initial period of rapid glycogenolysis, the slower rate of glycogen breakdown was balanced by glycogen synthesis from glucose. This amounted to about 20% of the glycogen decrease during the first 3 minutes of epinephrine action. When acetate was present in the perfusate instead of glucose, no glycogen synthesis occurred, and the total glycogen decrease over the 30-minute perfusion period in the presence of epinephrine was correspondingly greater.

The present investigation shows that the major glycogenolytic effect of epinephrine was of brief duration compared with the sustained stimulation of glucose and acetate oxidation. Low concentrations of epinephrine or norepinephrine (10^{-7} M) increased glucose uptake 2-fold, and increased the heart rate but did not increase lactate formation or glycogenolysis. Dichloroisoproterenol abolished the glycogenolytic effect of 10^{-6} M epinephrine, but did not decrease the elevated glucose uptake to control levels. Fluorescence studies with intact rat heart (54) have established that epinephrine in the concentration range of 10^{-7} M produces a transient glycogenolysis, as shown by a brief cycle of reduction of cytoplasmic pyridine nucleotide followed by reoxidation, even though net glycogen or lactate changes could not be detected. The effect of epinephrine on the mechanical activity of the heart was considerably more prolonged. These results suggest a temporal dissociation of the glycogenolytic effect of epinephrine from the stimulatory effect on oxidative metabolism. A similar relationship between activation of phosphorylase and the inotropic action of epinephrine has recently been reported by Mayer, Cotten, and Moran with dog hearts *in vivo* (46). These observations, in contrast to those of Hess *et al.* (41–43), indicate that activation of phosphorylase with consequent glycogenolysis, and the increased work of the heart, represent separate effects of epinephrine. This conclusion is supported by the experiments of Vincent and Ellis (44) on isolated guinea pig hearts, which showed that acetylcholine decreased the glycogenolytic effect of epinephrine to a much greater extent than the inotropic effect.

Sutherland and Rall (6, 55) have reviewed evidence showing that epinephrine exerts its glycogenolytic effect by increasing the tissue levels of adenosine 3′,5′-phosphate (cyclic AMP). The basis of the inotropic action of catecholamines is not known at present, and speculation that cyclic AMP is involved is based on indirect evidence (6). It would be of great interest to know whether tissue levels of cyclic AMP after epinephrine stimulation follow the relatively transient pattern of phosphorylase activation or the more prolonged time course of increased mechanical activity and fuel utilization. If cyclic AMP is indeed the mediator of both the glycogenolytic and the inotropic actions of epinephrine, the contractile elements of cardiac muscle may be more sensitive than the phosphorylase-activating system. The similarity of the effects of glucagon (30, 56, 57) on the heart with those of epinephrine supports the concept (7) that these agents exert their effects by a common mechanism.

SUMMARY

1. Epinephrine (10^{-6} M) produced a 3- to 4-fold, approximately linear, increase of glucose uptake by the perfused rat heart over a period of 30 minutes. Lactate production increased 8-fold, and glycogen decreased by 45%, primarily within the first 5 minutes of epinephrine addition. Dichloroisoproterenol (0.5 μg per ml) abolished the glycogenolytic effect of epinephrine, and inhibited the stimulation of glucose uptake by 50%.

2. Norepinephrine was equipotent with epinephrine in producing the metabolic changes, and isoproterenol was more potent. Low concentrations of epinephrine or norepinephrine (10^{-7} M) increased glucose uptake 2-fold, but did not cause appreciable glycogen or lactate changes.

3. The phosphorylase *a* activity of rat heart increased from 8 to about 70% of total phosphorylase within 20 seconds of epinephrine addition, and decreased to 25% of total phosphorylase after 10 minutes. Glycogenolysis and lactate production approximately paralleled phosphorylase *a* activity, and glycogen was shown to be the major source of medium lactate.

4. Insulin doubled $^{14}CO_2$ production from uniformly labeled glucose-^{14}C, and epinephrine substantially increased glucose oxidation to $^{14}CO_2$ in both the presence and absence of insulin. The incorporation of ^{14}C-glucose into glycogen was stimulated by epinephrine despite a decrease of total cardiac glycogen.

5. The uptake and oxidation of acetate-2-^{14}C and the release of glycerol into the perfusate were also increased by epinephrine over a 30-minute perfusion period, showing that endogenous and exogenous noncarbohydrate precursors could be used instead of glucose to support the increased mechanical activity.

6. The present studies show that epinephrine causes an increased fuel utilization, either in the absence of demonstrable glycogen change, or when glycogen breakdown had ceased. The results suggest a dual action of epinephrine: an initial transient stimulation of glycogenolysis followed by a more prolonged increase of oxidative metabolism. It is proposed that the increased oxidative metabolism is a direct consequence of increased mechanical activity.

REFERENCES

1. Ellis, S., *Pharmacol. Revs.*, **8**, 486 (1956).
2. De Bodo, R. C., and Altszuler, N., *Physiol. Revs.*, **38**, 389 (1958).
3. Dickman, S. R., Wiest, W. G., and Eik-Nes, K., *Am. J. Physiol.*, **194**, 327 (1958).
4. Fritz, I. B., Shatton, J., Morton, J. V., and Levine, R., *Am. J. Physiol.*, **189**, 57 (1957).
5. Ellis, S., *Pharmacol. Revs.*, **11**, 469 (1959).
6. Sutherland, E. W., and Rall, T. W., *Pharmacol. Revs.*, **12**, 265 (1960).
7. Sutherland, E. W., *The Harvey Lectures, Ser. 57 (1961–1962)*, 17 (1962).
8. Drury, D. R., and Wick, A. N., *Am. J. Physiol.*, **194**, 465 (1958).
9. Bloom, W. L., and Russell, J. A., *Am. J. Physiol.*, **183**, 356 (1955).
10. Mayer, S. E., *Biochem. Pharmacol.*, **12**, 193 (1963).
11. Hornbrook, K. R., and Brady, T. M., *J. Pharmacol. Exptl. Therap.*, **140**, 295 (1963).
12. Randle, P. J., and Morgan, H. E., in R. S. Harris and I. G. Wool (Editors), *Vitamins and hormones, Vol. 20*, Academic Press, Inc., New York, 1962, p. 199.
13. Kipnis, D. M., Helmreich, E., and Cori, C. F., *J. Biol. Chem.*, **234**, 165 (1959).
14. Belford, J., and Feinleib, M. R., *Biochem. Pharmacol.*, **11**, 987 (1962).
15. Patterson, S. W., and Starling, E. H., *J. Physiol. (London)*, **47**, 137 (1913).
16. Evans, C. L., and Ogawa, S., *J. Physiol. (London)*, **47**, 446 (1913).
17. Bogue, J. Y., Evans, C. L., Grande, F., and Hsu, F. Y., *Quart. J. Exptl. Physiol.*, **25**, 213 (1935).

18. Dill, D. B., Edwards, H. T., and deMeio, R. H., *Am. J. Physiol.*, **111**, 9 (1935).
19. Ellis, S., Davis, A. H., and Anderson, H. L., *J. Pharmacol. Exptl. Therap.*, **115**, 120 (1955).
20. Century, B., *Federation Proc.*, **13**, 342 (1954).
21. Herrmann, R. G., Flamboe, G. E., and Chen, K. K., *J. Pharmacol. Exptl. Therap.*, **112**, 23 (1954).
22. Walaas, O., and Walaas, E., *J. Biol. Chem.*, **187**, 769 (1950).
23. Fisher, R. B., and Williamson, J. R., *J. Physiol.* (*London*), **158**, 102 (1961).
24. Lee, K. S., *J. Pharmacol. Exptl. Therap.*, **109**, 313 (1953).
25. Williamson, J. R., and Kreisberg, R. A., *Federation Proc.*, **22**, 288 (1963).
26. Williamson, J. R., and Krebs, H. A., *Biochem. J.*, **80**, 540 (1961).
27. Bleehen, N. M., and Fisher, R. B., *J. Physiol.* (*London*), **123**, 260 (1954).
28. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric techniques and tissue metabolism*, Burgess Publishing Company, Minneapolis, 1949.
29. Fisher, R. B., and Williamson, J. R., *J. Physiol.* (*London*), **158**, 86 (1961).
30. Cornblath, M., Randle, P. J., Parmeggiani, A., and Morgan, H. E., *J. Biol. Chem.*, **238**, 1592 (1963).
31. Opie, L. H., Shipp, J. C., Evans, J. R., and Leboeuf, B., *Am. J. Physiol.*, **203**, 839 (1962).
32. Wollenberger, A., Ristau, O., and Schoffa, G., *Arch. ges. Physiol., Pflüger's*, **270**, 399 (1960).
33. Sutherland, E. W., and Wosilait, W. D., *J. Biol. Chem.*, **218**, 459 (1956).
34. Huggett, A. St. G., and Nixon, D. A., *Lancet*, **ii**, 368 (1957).
35. Horn, H. D., and Bruns, F. H., *Biochim. et Biophys. Acta*, **21**, 378 (1956).
36. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **100**, 485 (1933).
37. Garland, P. B., and Randle, P. J., *Nature*, **196**, 987 (1962).
38. Danforth, W. H., Helmreich, E., and Cori, C. F., *Proc. Natl. Acad. Sci. U. S.*, **48**, 1191 (1962).
39. Lowry, O. H., and Lopez, J. A., *J. Biol. Chem.*, **162**, 421 (1946).
40. Haugaard, N., Hess, M. E., Shanfeld, J., Inesi, G., and Kukovetz, W. R., *J. Pharmacol. Exptl. Therap.*, **131**, 137 (1961).
41. Kukovetz, W. R., Hess, M. E., Shanfeld, J., and Haugaard, N., *J. Pharmacol. Exptl. Therap.*, **127**, 122 (1959).
42. Hess, M. E., Shanfeld, J., and Haugaard, N., *J. Pharmacol. Exptl. Therap.*, **135**, 191 (1962).
43. Hess, M. E., Shanfeld, J., and Haugaard, N., *Biochem. Pharmacol.*, **11**, 1031 (1962).
44. Vincent, N. H., and Ellis, S., *J. Pharmacol. Exptl. Therap.*, **139**, 60 (1963).
45. Mayer, S. E., and Moran, N. C., *J. Pharmacol. Exptl. Therap.*, **129**, 271 (1960).
46. Mayer, S. E., Cotten, M. de V., and Moran, N. C., *J. Pharmacol. Exptl. Therap.*, **139**, 275 (1963).
47. Moran, N. C., and Cotten, M. de V., in G. E. Bourne (Editor), *Structure and function of muscle, Vol. III*, Academic Press, Inc., New York, 1960, p. 31.
48. Williamson, J. R., *Biochem. J.*, **83**, 377 (1962).
49. Shipp, J. C., Thomas, J. M., and Crevasse, L., *Science*, **143**, 371 (1964).
50. Neptune, E. M., Jr., Sudduth, H. C., Foreman, D. R., and Fash, F. J., *J. Lipid Research*, **1**, 229 (1960).
51. Randle, P. J., Garland, P. B., Hales, C. N., and Newsholme, E. A., *Lancet*, **i**, 785 (1963).
52. Vaughan, M., *J. Lipid Research*, **2**, 293 (1961).
53. Williamson, J. R., *Federation Proc.*, **21**, 320 (1962).
54. Williamson, J. R., and Chance, B., *Abstracts of the VIth International Congress of Biochemistry*, New York, 1964, p. 736.
55. Rall, F. W., and Sutherland, E. W., *Cold Spring Harbor symposia on quantitative biology, Vol. 26*, Long Island Biological Association, Cold Spring Harbor, Long Island, New York, 1961, p. 347.
56. Farah, A., and Tuttle, R., *J. Pharmacol. Exptl. Therap.*, **129**, 49 (1960).
57. Kreisberg, R. A., and Williamson, J. R., *Am. J. Physiol.*, in press.

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Purification and Properties of the Transglucosylase Inhibitor of *Mycobacterium tuberculosis**

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We have previously presented data (1) on the presence, in cell-free extracts of *Mycobacterium tuberculosis*, strain H37Ra, of an inhibitor of a uridine diphosphate glucose:D-glucose 6-phosphate 1-glucosyltransferase (transglucosylase). In the presence of this inhibitor the synthesis of trehalose 6-phosphate from uridine diphosphate glucose and glucose 6-phosphate is blocked. The inhibitor has now been highly purified and its properties have been investigated. This report presents our results leading to the conclusion that the inhibitor is a low molecular weight oligoribonucleotide containing adenine and guanine.

EXPERIMENTAL PROCEDURE

The sources of materials and details of methods not otherwise described in this paper are the same as reported earlier (1). RNase (recrystallized five times) was obtained from the Sigma Chemical Company.

Methods—Phosphate was determined by the method of Fiske and SubbaRow (2), and of King (3), and ribose by the orcinol method of Brown (4). Analysis of acid digests of oligonucleotides for pyrimidine ribose was performed by the bromination method of Massart and Harte (5). Guanylic acid was routinely employed as an analytical standard. Pyrimidine ribotides were separated from purines following acid hydrolysis by the method of Bergmann and Dikstein (6). The chromatography of RNA digests on urea-DEAE-cellulose (7) columns has been described (8, 9). Purines were identified by paper chromatography followed by ultraviolet spectrophotometry (10).

The assay systems for the transglucosylase and the transglucosylase inhibitor were the same as those described earlier (1), except that Co^{++} at a final concentration of 0.1 mM was incorporated in the latter assay system. The reason for this requirement of Co^{++} when the pure transglucosylase inhibitor is assayed is not clear.

RESULTS

Experiments with Crude Transglucosylase Inhibitor—The crude inhibitor of the transglucosylase is destroyed by storage of a phosphate-buffered, crude, cell-free extract either at −15° for several months, or at 2° for 24 hours. Dialysis of the crude, cell-free extract against phosphate buffer destroys the inhibitor; dialysis against water or EDTA is without effect. Destruction of the inhibitor in these crude cell-free extracts is accompanied by an increase in transglucosylase activity (Table I). Since the partially purified transglucosylase is stable when dialyzed against phosphate buffer (Table I), the increase in transglucosylase activity of the crude extract cannot be attributed to an activation of the transglucosylase by phosphate.

The destruction of the transglucosylase inhibitor in the presence of phosphate is accompanied by an increase in the ultraviolet absorption of the extract (or the dialysis fluid); the increase is maximal at 260 to 270 mμ.

These results suggested that the inhibitor might act as a substrate for a nucleoside diphosphate:polynucleotide nucleotidyltransferase (polynucleotide phosphorylase). Previous experiments had shown the presence of large amounts of polynucleotide phosphorylase in cell-free extracts of H37Ra. The polynucleotide phosphorylase is partially inactivated at 37° in the presence of phosphate and magnesium; at 2° it is unaffected by these ions. The data shown in Table II indicate that in crude cell-free extracts the destruction of the transglucosylase inhibitor is reduced as the polynucleotide phosphorylase is inactivated.

The crude inhibitor is not dialyzable at low salt concentrations. However, the inhibitor passes across the dialysis membrane when dialysis is carried out against either 1 M KCl or 1 M $(NH_4)_2CO_3$.

Attempts to purify the transglucosylase inhibitor were based on the tentative conclusion, reached as a result of these experiments, that the inhibitor is a polynucleotide.

Purification of Transglucosylase Inhibitor—Two techniques were found to be applicable to the purification of the transglucosylase inhibitor. The first, based on the chloroform method of Sevag, Lachman, and Smolens (11), yielded a partially purified transglucosylase inhibitor contaminated with some protein. The second method was based on modifications of the phenol method of Kirby (12) and of Gierer and Schramm (13). The modifications in the procedure consisted of the following. (*a*) Excess phenol was removed from the aqueous solution of soluble ribonucleic acid by ethyl ether. (*b*) Instead of the use of one-step ethyl alcohol precipitation of the sRNA,[1] the aqueous solution was fractionated with ethyl alcohol; the fraction precipitating between 45 and 75% of ethyl alcohol (by volume) was recovered, washed with 80% ethyl alcohol, and dissolved in distilled water. From each milliliter of crude cell-free extract, 2 mg of sRNA powder (75% of the transglucosylase inhibitor activity) were

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[1] The abbreviation used is: sRNA, soluble ribonucleic acid.

TABLE I

Activation of transglucosylase of H37Ra

Treatment	Specific activity*
Transglucosylase preparation, crude extract	
0.001 M EDTA, 2°, 6 days	0.0
0.001 M EDTA, dialyzed, 2°, 24 hrs	0.1
0.02 M PO_4^{3-}, 2°, 6 days	0.50
0.02 M PO_4^{3-}, dialyzed, 2°, 24 hrs	0.65
Transglucosylase preparation, partially purified	
None, fresh preparation	6.3
0.002 M EDTA, dialyzed, 2°, 24 hrs	5.8
0.02 M PO_4^{3-}, dialyzed, 2°, 24 hrs	5.8

* Millimicromoles of trehalose 6-phosphate formed per minute per mg of protein.

TABLE II

Association of polynucleotide phosphorylase with destruction of transglucosylase inhibitor

The crude cell-free extract of *M. tuberculosis* was dialyzed for 18 hours against 0.001 M $KHCO_3$ and divided into three samples. Sample 1 was heated at 65° for 10 minutes in the presence of 0.02 M phosphate and 0.002 M $MgCl_2$ (final pH, 7.5). Sample 2 was heated to 37° for 30 minutes in this same buffer. Samples 1 and 2, together with untreated Sample 3, were dialyzed for 18 hours at 2° against 0.02 M phosphate buffer, pH 7.5, that was also 0.002 M in $MgCl_2$. The dialyzed samples were assayed for their content of polynucleotide phosphorylase and of transglucosylase inhibitor.

Sample	Polynucleotide phosphorylase activity*	Inhibitor content†
1	0.0	21.0
2	30.0	16.0
3	90.0	6.4

* Millimicromoles of nucleoside diphosphate formed per minute per ml of sample assayed against endogenous RNA.

† Transglucosylase activity (expressed as millimicromoles of UDP formed per minute per mg of protein) removed per ml of sample.

recovered. Chromatography of this material on DEAE-cellulose with urea (7 M)-NH_4HCO_3 (0.02 M to 0.6 M gradient) elution yielded two fractions showing ultraviolet absorption (Fig. 1). The first fraction, at 0.15 M NH_4HCO_3, was devoid of inhibitor activity; the second fraction, at about 0.4 M NH_4HCO_3, contained all the inhibitor. Results of a typical purification procedure are shown in Table III.

This chromatographic behavior of the transglucosylase inhibitor suggests the presence of no fewer than 6, and no more than 9, bases per molecule (9).

The ultraviolet absorption spectrum of the transglucosylase inhibitor is shown in Fig. 2.

Chemical Analysis of Transglucosylase Inhibitor—The purified transglucosylase inhibitor was hydrolyzed in 1 M HCl for 60 minutes at 100°. The hydrolyzed material was analyzed for purine bases and pyrimidine ribonucleotides as described above. Chromatographic analysis of the purine fraction showed the presence of adenine and guanine; spectrophotometric confirmation of these bases was obtained.

No evidence could be obtained for the presence of pyrimidine derivatives. Chromatography (14) of the acid digest following the procedure for separation of purines from pyrimidine ribonucleotides (6) yielded, in the latter fraction, only a diffuse, non-purine-containing, ultraviolet-absorbing spot which did not stain with silver-bromphenol blue (15). Rechromatography of an eluate of this spot in Pabst System II (16) gave no indication of the presence of pyrimidine derivatives. This non-pyrimidine-containing, ultraviolet-absorbing material appears to be an artifact of the acid hydrolysis-mercury separation procedure, for it is found when pure guanylic acid is subjected to these procedures; ordinary acid hydrolysis of guanylic acid yields none of this material.

The data of Table IV present the analytical evidence that the transglucosylase inhibitor is a polyribonucleotide containing 2 moles of guanine per mole of adenine.

Evidence that Transglucosylase Inhibitor Is Not Produced by Random Degradation of Mycobacterial RNA—The suggestion for uniqueness of the inhibitory polyribonucleotide demands the demonstration (*a*) that this inhibitor must have a specific struc-

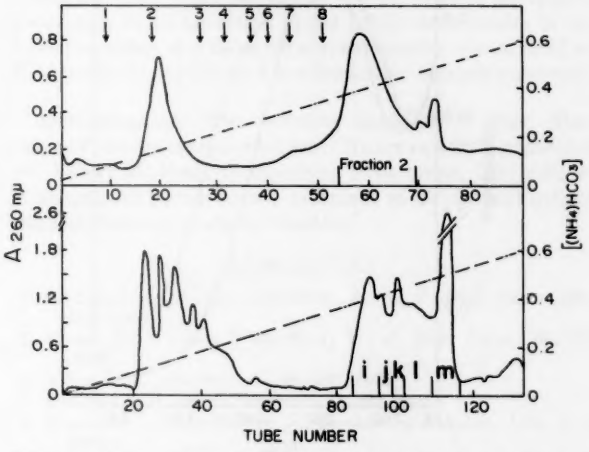

FIG. 1. *Upper:* chromatography of the transglucosylase inhibitor on urea-DEAE-cellulose. *Solid line*, absorbance at 260 mμ; *dashed line*, molarity of NH_4HCO_3 eluate (pH 8.3). *Numbered arrows* denote elution positions of known oligoribonucleotides (9). These numbers correspond to net negative charges per molecule. *Lower:* chromatography of RNase digest of sRNA from *M. tuberculosis*. Analytical conditions as described for the *upper figure*. After dialysis first against 1 M KCl, and then against distilled water, 400 absorbance units of sRNA (0 to 45% ethanol fraction as described above) were digested with 0.50 mg of pancreatic RNase. The mixture was incubated at 37°; pH was maintained at 8 by the addition of 5 M NH_4OH. Incubation time was 180 minutes.

TABLE III

Purification of transglucosylase inhibitor

Purification step	Volume	Absorbance at 260 mμ	Inhibitor content	Specific activity*
	ml		*units*	
Crude extract	450	100,000	3,500	0.035
Phenol extract	500	10,000	2,000	0.20
Alcohol fractionation	10	200	800	4.0
Eluate from DEAE-cellulose column	10	100	600	6.0

* Inhibitor units per unit of absorbance at 260 mμ.

ture, and (*b*) that its occurrence in cell-free extracts must be independent of enzymatic degradation of cellular RNA. In the preparation of the transglucosylase inhibitor, sRNA is removed by precipitation with ethyl alcohol (0 to 45 volume %) before the transglucosylase inhibitor is precipitated. Soluble ribonucleic acid prepared in this fashion and dialyzed against 1 M KCl characteristically shows transglucosylase inhibitor activity (30% of the whole extract inhibitor activity). The mycobacterial sRNA was hydrolyzed with RNase; the transglucosylase inhibitor is unaffected by this RNase treatment. The hydrolyzed sRNA preparation had the same transglucosylase inhibitor content as did the unhydrolyzed sRNA. Upon chromatography of the hydrolyzed sRNA preparation (Fig. 1), transglucosylase inhibitor activity appeared in the peak predicted from previous experiments with the pure transglucosylase inhibitor and in only that quantity measured before chromatography (Table V). The production of oligonucleotides with the same number of base units as the transglucosylase inhibitor had no effect on the amount of the inhibitor originally present as a contaminant of the sRNA.

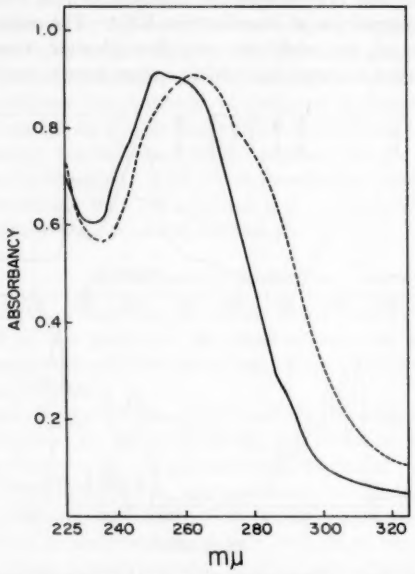

FIG. 2. Ultraviolet absorption spectrum of the transglucosylase inhibitor in 20 mM phosphate buffer, pH 7.0 (*solid line*), and in 10 mM HCl (*dashed line*). The concentration of the transglucosylase inhibitor was the same in both experiments.

TABLE IV

Nucleotide composition of transglucosylase inhibitor

The contents of the center tubes of the 7 M urea-0.40 M NH_4HCO_3 eluate from the DEAE-cellulose column were combined and analyzed.*

Component	Concentration
	μmole/ml
Adenine	0.15
Guanine	0.31
Adenine + guanine	0.46 ± 0.02
Ribose	0.49 ± 0.03
Phosphate	0.45 ± 0.03

* The nonpurine, ultraviolet-absorbing contaminant (see text) present in this fraction is removed in the course of the pyrimidine ribonucleotide analysis (6) and consequently does not interfere with this analysis.

TABLE V

Recovery of transglucosylase inhibitor from ribonuclease digest of soluble mycobacterial ribonucleic acid

The conditions are as described in the legend of Fig. 1 (*lower*). After RNase digestion, 42 inhibitor units and 220 absorbance units were placed on the column.

Fraction	Inhibitor	Absorbance
	units	*units*
sRNA	65	330
RNase digest of sRNA	62	
Column fractions*		
i	20	30
j	3.0	4.3
k	2.9	22.5
l	3.0	31.0
m	4.2	36.0
Total	33.1	123.8

* Refer to Fig. 1 (*lower*).

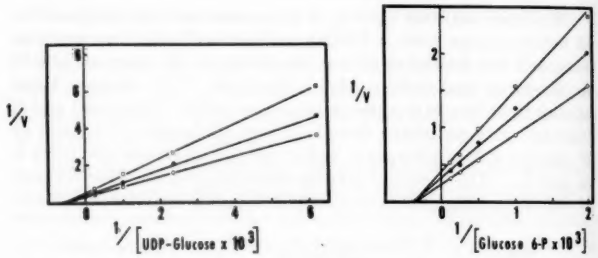

FIG. 3 (*left*). Noncompetitive inhibition of the H37Ra transglucosylase by the purified transglucosylase inhibitor. The standard one-step transglucosylase assay system (1) was used except that the UDP-glucose was varied as shown. Each reaction mixture contained 140 units of partially purified transglucosylase and the following: ○——○, no inhibitor; ●——●, 22 units of inhibitor; and □——□, 44 units of inhibitor. Velocity is defined as millimicromoles of UDP formed per minute.

FIG. 4 (*right*). The conditions are the same as for Fig. 3 except that glucose 6-phosphate was varied as shown. ○——○, no inhibitor; ●——●, 22 units of inhibitor; □——□, 44 units of inhibitor.

Type of Inhibition of Transglucosylase—Figs. 3 and 4 show that the transglucosylase inhibitor acts noncompetitively with the transglucosylase (1).

DISCUSSION

The conclusion that may be drawn from the data reported above is that cell-free extracts of H37Ra contain a low molecular weight oligoribonucleotide which acts as a noncompetitive inhibitor of a transglucosylase. We have shown this inhibitor to be specific (*a*) in terms of its differentiation from sRNA; (*b*) by its absence from the products of hydrolysis of sRNA; and (*c*) by its action on the transglucosylase and not on any other enzyme in the assay system.

Several observations remain to be explained. The transglucosylase inhibitor has little effect on the transglucosylase of physiologically young cultures of mycobacteria (1). In addition, this inhibitor is without effect on the transglucosylase of yeast (1). Both of these points may be resolved by a recent observation, which will shortly be reported in detail. We have now found that the transglucosylase of physiologically old H37Ra cultures can be reversibly converted to a form which, although catalytically fully active, is unaffected by the transglucosylase inhibitor.

Although there are many examples of the activation of enzymes through removal of fragments of low molecular weight (*e.g.* activation of chymotrypsinogen (17–19) and trypsinogen (20–22)) and many examples of inhibition of enzymes (*e.g.* trypsin (23–27)) by compounds of low molecular weight, these effects differ from the activation and inhibition of the mycobacterial transglucosylase in that they represent either an irreversible activation following cleavage of specific peptide bonds, or the competitive inhibition of an enzyme by a low molecular weight protein.

The latent RNase (28, 29) and DNase (29) of *Escherichia coli* are examples of an enzyme-inhibitor complex similar to the transglucosylase-transglucosylase inhibitor complex described above. These enzymes are activated only when the bacterial ribonucleoprotein is cleaved into its nucleic acid and protein components (28, 29).

The transglucosylase inhibitor could be produced in one of the following ways.

1. This oligoribonucleotide could represent a synthetic end product that has no metabolic significance but happens to inhibit an enzyme.

2. The transglucosylase inhibitor could be derived from the RNA of the enzyme-forming system which catalyzes the formation of the transglucosylase. It is possible that when the newly synthesized protein leaves the enzyme-forming system it carries with it, at the active center of the newly synthesized enzyme molecule, a piece of the RNA of the enzyme-forming system. This oligoribonucleotide, coded for the amino acid sequence of the active center, would be an extremely effective inhibitor of the enzyme.

3. Alternatively, the transglucosylase inhibitor could be a chance product of RNA degradation. As the enzyme-forming system is turned over, the RNA is degraded and resynthesized. If an oligoribonucleotide corresponding to the structure of the transglucosylase inhibitor exists within a molecule of RNA, then this segment might be an end product of RNA degradation and, in this fashion, accumulate within the cell. We feel that this alternative may be ruled out by the lack of formation of the transglucosylase inhibitor following hydrolysis of sRNA by RNase.

In any event, we assume the formation, in the avirulent (H37Ra) strain of *M. tuberculosis*, of an oligoribonucleotide[2] which, is not formed in the virulent (H37Rv) strain of this organism. Since the avirulent strain resulted from a single mutation of the virulent strain, then the presence of this oligoribonucleotide could be explained by a single alteration in the DNA which, in turn, leads to an altered RNA, either soluble or ribosomal.

[2] We have assigned the trivial name, Mycoribnin, to this transglucosylase inhibitor. A United States patent has been applied for Mycoribnin by the authors and has been assigned to the United States Government under terms of Executive Order 10096.

SUMMARY

Cell-free extracts of the H37Ra (avirulent) strain of *Mycobacterium tuberculosis* contain a masked transglucosylase. An inhibitor of the transglucosylase has been extensively purified. When freed of the inhibitor, the transglucosylase catalyzes the synthesis of trehalose 6-phosphate from uridine diphosphate glucose and glucose 6-phosphate.

The transglucosylase inhibitor is an oligoribonucleotide containing between 6 and 9 purine bases and no pyrimidine bases. The oligoribonucleotide contains guanine and adenine in a molar ratio of 2:1. The transglucosylase inhibitor noncompetitively inhibits the transglucosylase, and is distinct from, and is not produced by ribonuclease digestion of, soluble ribonucleic acid. The relation of this inhibitor to latent enzymes is discussed.

It is suggested that the inhibitory oligoribonucleotide is either a portion of the enzyme-forming system which accompanies the newly synthesized protein molecule, or is a portion of an RNA molecule which cannot be hydrolyzed during normal RNA turnover.

The mutation which led to the H37Ra strain is interpreted accordingly as an alteration in the DNA which results in the formation either of a defective enzyme-forming system or of an RNA molecule containing a nondegradable oligoribonucleotide.

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REFERENCES

1. Goldman, D. S., and Lornitzo, F. A., *J. Biol. Chem.*, **237**, 3332 (1962).
2. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
3. King, E. J., *Biochem. J.*, **26**, 292 (1932).
4. Brown, A. H., *Arch. Biochem.*, **11**, 269 (1946).
5. Massart, L., and Harte, J., *Biochim. et Biophys. Acta*, **1**, 83 (1947).
6. Bergmann, F., and Dikstein, S., in D. Glick (Editor), *Methods of biochemical analysis; Vol. 6*, Interscience Publishers, Inc., New York, 1958, p. 79.
7. Peterson, E. A., and Sober, H. A., *J. Am. Chem. Soc.*, **78**, 75 (1956).
8. Tomlinson, R. V., and Tener, G. M., *J. Am. Chem. Soc.*, **84**, 2644 (1962).
9. Lipsett, M. N., and Heppel, L. A., *J. Am. Chem. Soc.*, **85**, 118 (1963).
10. Loring, H. S., Fairley, J. L., Bortner, H. W., and Seagran, H. L., *J. Biol. Chem.*, **197**, 809 (1952).
11. Sevag, M. G., Lachman, D. B., and Smolens, J., *J. Biol. Chem.*, **124**, 425 (1938).
12. Kirby, K. S., *Biochem. J.*, **64**, 405 (1956).
13. Gierer, A., and Schramm, G., *Nature*, **177**, 702 (1956).
14. Carter, C. E., *J. Am. Chem. Soc.*, **72**, 1466 (1950).
15. Wood, T., *Nature*, **176**, 175 (1955).
16. *Circular OR-17*, Pabst Laboratories, Division of Pabst Brewing Company, Milwaukee, 1961.
17. Rovery, M., Poilroux, M., Curnier, A., and Desnuelle, P., *Biochim. et Biophys. Acta*, **16**, 590 (1955).
18. Bettelheim, F. R., and Neurath, H., *J. Biol. Chem.*, **212**, 241 (1955).
19. Rovery, M., Poilroux, M., Yoshida, A., and Desnuelle, P., *Biochim. et Biophys. Acta*, **23**, 608 (1957).
20. Davie, E. W., and Neurath, H., *J. Biol. Chem.*, **212**, 515 (1955).

21. Gabeloteau, C., and Desnuelle, P., *Arch. Biochem. Biophys.*, **69**, 475 (1957).
22. Neurath, H., and Dixon, G. H., *Federation Proc.*, **16**, 791 (1957).
23. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **19**, 991 (1936).
24. Kunitz, M., *J. Gen. Physiol.*, **30**, 291 (1947).
25. Lineweaver, H., and Murray, C. W., *J. Biol. Chem.*, **171**, 565 (1947).
26. Fraenkel-Conrat, H., Bean, R. C., Ducay, E. D., and Olcott, H. S., *Arch. Biochem. Biophys.*, **37**, 393 (1952).
27. Laskowski, M., Jr., Mars, P. H., and Laskowski, M., *J. Biol. Chem.*, **198**, 745 (1952).
28. Elson, D., *Biochim. et Biophys. Acta*, **27**, 216 (1958).
29. Elson, D., *Biochim. et Biophys. Acta*, **36**, 372 (1959).